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13. ABSTRACT *(Maximum 200 Words)*

Estrogen receptor (ER) is ligand-dependent transcription factor that has an important role in the development and progression of breast cancer. In this study, we developed a chimeric repressor to turn off ER target genes with the aim to directly investigate the role of ER target genes in tumor progression. The chimeric repressor contains the ER DNA-binding domain, a Krupple-Associated Box (KRAB) repressor domain and a truncated progesterone ligand-binding domain. The ability of the chimeric repressor to block ER mediated transcription was assessed in transient transfection assays. ER-induced reporter activity was inhibited by the repressor in a dose-dependent manner, with the maximum effect of more than 80% reduction. The inhibitory activity of the chimeric repressor was tightly under the control of RU486. Though we successfully generated regulable repressors to inhibit the ER reporter genes *in vitro* in transient transfection, these repressors fail to suppress endogenous ER target gene expression in breast cancer cell lines. More efforts need to be focus on in vivo gene expression. The success in creating such a repressor will provide a useful tool to study the role of ER target genes in breast cancer progression, and it may be potentially useful for gene therapy of breast cancer.

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Introduction

Proper function of estrogen receptor is critical for normal mammary gland development. It is conceivable that cells with a genetic alteration in favor of hormone stimulation would gain a growth advantage and contribute the initiation and maintenance of breast cancer before progressing into a more aggressive stage.

ER belongs to a large family of nuclear receptors that function as ligand-inducible transcription factors. As a feature of nuclear receptors, ER is composed of three major functional domains: a ligand-binding domain, a DNA binding domain and a transactivation domain. Upon binding to cognate ligand, ER is activated, dimerizes and binds to specific DNA response elements (EREs) in the regulatory region of target genes, eventually stimulating target gene expression [1, 2]. Among ER target genes are growth factors that act as direct mitogens in a paracrine or autocrine manner to stimulate epithelial proliferation. More than half of breast carcinomas are found to be ER positive and retain some degree of steroid responsiveness [3]. An increased level of ER expression in breast cancer tissue implies that the tumor could have arisen from a subset of cells or ER positive cells could be more vulnerable for oncogenic challenge.

The antiestrogen, tamoxifen, has been developed to block the binding of estrogen to its receptor. Tamoxifen has been used successfully to inhibit ER dependent growth of breast cancer [4]. A major unresolved issue is that why most breast cancers that contain ER eventually become resistant to estrogen-ablation therapy [5]. It has been proposed that the mutation of ER to a constitutively active receptor or to a receptor which can be activated by estrogen antagonists, like tamoxifen or other steroidal compounds existing in the blood stream may contribute to the transition from estrogen-dependent to -independent tumor growth [6, 7]. If this hypothesis is correct, attempts to block the interaction between ER and estrogen by antagonists will not be able to suppress ER function. However, half of all advanced breast cancers are estrogen receptor positive but resistant to antiestrogen therapy. Some ER negative tumors behave as if they are ER positive in their expression of ER target genes such as the progesterone receptor. Furthermore, many of ER mutations identified in tumor cells are also found in healthy cells of breast cancer patients or healthy individuals. Thus, it remains controversial whether ER mutations have a primary role in the transition from estrogen-dependent to -independent states. An additional explanation is that the activation of ER may be possible through ligand independent pathway. Indeed, growth factors, intracellular protein kinases [8-10], the cell cycle regulator cyclin D1 and cyclin-dependent kinase (cdk2/cyclin A) complex [11-13] have been shown to modulate the activity of unliganded ER in different model systems. It is hypothesized that the unliganded receptor activation occurs via receptor phosphorylation. This phosphorylation leads to changes in receptor conformations whereby the potential to interact with co-activators or other transcription factors may be different from conformations induced by estrogens. This ligand-independent activity may also be enhanced by the addition of the partial agonist tamoxifen.

Though ER could be activated either by its ligand or alternatively by either mutations or ligand-independent pathways, the activated form(s) of ER will ultimately act on the regulatory region of its target genes to exert its biological function. In this study,

we constructed a regulable repressor which will bind to an ERE and silence the ER target gene expression in response to exogenous stimuli. A similar strategy has been employed to construct a regulable inducer (GLVP) that activates target gene expression in response to exogenous signal [14, 15]. To generate a repressor, we used the krupple-associated box (KRAB) that is a highly conserved repression domain in the krupple-class zinc finger family of transcription factors [16, 17]. When KRAB is linked to a heterologous DNA-binding domain, it can shut off transcription of target genes containing the DNA response element to which the chimeric protein binds [18]. Here we constructed a fusion protein linking KRAB to the estrogen receptor DNA-binding domain to repress all the ER target genes with ERE in their promoters. In order to generate a regulable repressor, we used a truncated ligand-binding domain of progesterone receptor which binds specifically to the antiprogestin, RU486 [19]. Upon binding of RU486, the inducible repressor will dimerize and bind to EREs to suppress the ER target gene expression as depicted in **Fig 1A**. Once the regulable repressor is expressed in tumor cells, the temporal repression of ER target genes can be closely regulated. The success of regulable repressor will have far reaching effect on defining the role of ER target genes in mammary gland ontogenesis and in the recurrence of tumor growth in an estrogen-independent manner.

Body

Generation of an inducible repressor

Based on the modular nature of transcription factors we generated chimeric repressors in attempt to specifically turn off the ER target genes in the presence of exogenous ligand. Regulable repressors contain a KRAB domain either at both N- and C-termini (KEDPK) or only at the C-terminus (EDPK), an ER DNA-binding domain and a truncated progesterone receptor ligand-binding domain (-19) as shown in **Fig 1B**. The KRAB domain we used is a conserved region of 75 amino acids present in the N-terminus of Kid-1, a member of the Krupple class of transcription factors isolated from rat kidney [21]. Fusion of this KRAB domain to DNA binding domains of the LacI/Z, Gal4 or TetR domains has been shown to be able to suppress the expression of respective reporters which contain corresponding binding site in their promoters [16-18]. Here, the KRAB domain was linked to an ER fragment (aa 175 to 282) comprising minimal ER DNA binding domain [22] to make sure that this construct can specifically bind to ERE with affinity compatible to the wild-type ER. The truncated PR ligand binding domain has been found to activate rather than repress receptor in the presence of antagonist RU486 [19]. Fusion of this mutated ligand binding domain to heterologous protein has shown to render it under the control of exogenous ligand RU486 [14, 15]. The specific feature of these regulable repressors is that they should only inhibit the target genes containing an ERE in the presence of RU486.

To ensure that correct proteins are made from the regulable repressor, we performed *in vitro* transcription/translation in parallel with the parental cloning vector and ER expression vector (66 KDa) as controls. As shown in **Fig 2**, no protein was translated from the empty vector. The expected size of translated proteins was produced

by EDPK and KEDPK expression vectors. These data suggested that the recombinant DNA constructs were able to express the full-length chimeric proteins.

The inducible repressor specifically inhibits ER dependent transcription in the presence of progesterone antagonist, RU486

To test the functional properties of the repressor, HeLa cells were co-transfected with repressor KEDPK and $(ERE)_3$TATA-Luc reporter plasmids together with a human ER expression vector. Luciferase activity in response to a saturating dose of E_2 (10^{-9} M) and RU486 (10^{-8} M) was measured in the absence or presence of repressor plasmids. As shown in **Fig 3A**, there is a seven-fold increase in ER dependent transcription in the presence of E_2. ER dependent transcription was not affected by addition of RU486. The chimeric repressor KEDPK has no significant effect on the reporter gene expression in the absence of exogenous ligand, RU486. However, KEDPK could effectively inhibit more than 80% of ER-mediated transcription in the presence of RU486 (10 nM). Thus, KEDPK could compete effectively for the ERE binding site with wild-type ER to suppress the transcription. EDPK could also inhibit ER induced transcription but was less potent than that of KEDPK (data not shown).

The specificity of repressor activity was assessed by co-transfection with reporter plasmid containing glucocorticoid response element (GREtk-Luc) which is palindromic DNA sequences similar to the ERE. Since RU486 has been reported to have glucocorticoid receptor (GR) antagonistic property, a constitutively active form of GR (GR*) with ligand binding domain truncation was used to exclude the effect of RU486 on GR activity [23]. The truncated GR (GR*) stimulated GR reporter gene expression over 50 fold. The repressor has no effect on the GR* induced transcription either in the absence or in the presence of RU486 (**Fig 3B**). Thus, the repressor specifically inhibited ER mediated transcription in transient transfection assays.

Dose-dependent inhibition of ER-mediated transcription by the inducible repressor

To further characterize the potency of the repressor on ER-dependent transcription, different amounts of KEDPK plasmid were co-transfected with the ER expression vector. Results shown in **Fig 4** indicate that inhibitory effect of the repressor on ER transcription was dose-dependent. A 50% reduction of ER-mediated transcription was observed when equal amounts of ER and repressor KEDPK plasmids were cotransfected. Maximum inhibition of the ER mediated transcription was observed when the amount of repressor was in four-fold excess that of ER. Further increases in the amount of repressor has an inhibitory effect on the reporter even in the absence of RU486 (data not shown), implying that the excess repressor is binding to the ERE site of the reporter gene in the absence of exogenous added ligand. Next, we assessed the effective dose of RU486 in inducing the repressor activity. As shown in **Fig 5**, the repressor exhibited a RU486 dose-dependent regulation of suppressive activity. The maximum inhibition of ER mediated transcription appeared at concentration of 10 nM, which is below the concentration of RU486 required to antagonize any progesterone and glucocorticoid activity. Similar results were observed by co-transfection of repressor and

reporter plasmids into the breast cancer cell line MCF-7 (data not shown). These results suggested that RU486 could be used as ligand to regulate KEDPK repressor activity with minimal effects on other steroid hormones.

Inducible repressor that antagonizes ER mediated transcription is independent of cellular and promoter context

ER contains two transactivation domains, AF1 and AF2, which operate in a cell and promoter-specific manner to mediated ER action. Tamoxifen, the most widely used agent in endocrine therapy of breast cancer, acts as a partial agonist of ER in a cell type specific manner. The partial agonist activity of tamoxifen has been proposed to relate to its ability to activate the AF1 of ER [24]. To examine whether the repressor we developed is capable of inhibiting tamoxifen-activated transcription, the repressor plasmid and a $(ERE)_3$TATA-Luc reporter were transfected together with an ER expression vector into HepG2 (human heptocellular carcinoma) cells where AF1 activity of ER was proven to be dominant [25]. As shown in **Fig 6**, 4-hydroxy-tamoxifen (4OH-T) treatment of HepG2 cells resulted in an eleven-fold induction of ER mediated transcription, which is about 10% of the response elicited by E_2. The repressor KEDPK inhibited the 4HOT-induced ER activity in the presence of RU486 as efficiently as it inhibited E_2 induced activity.

In addition, the efficacy of repressor functions was examined on a natural estrogen responsive promoter. In this case we chose the estrogen responsive complement factor 3 (C3) promoter which contains putative ERE [26]. The E_2 was able to stimulate luciferase reporter expression from the natural C3 promoter (**Fig 7**). Transcription was almost completely blocked in cells transfected with repressor after treatment of RU486 (10 nM). Taken together, these results indicated that repressor KEDPK could block the ER activity independent of cellular and promoter context.

Establishment of estrogen-dependent and –independent breast cancer cell lines stably expressing KEDPK repressor

The repressor KEDPK has been shown to specifically inhibit the ER reporter activity in the presence of RU486, with maximal effect of more than 80% inhibition in transient transfection. We next tried to generate stable cell lines expressing the repressor KEDPK in order to study the role of estrogen receptor target genes in breast cancer cell growth. The repressor KEDPK was subcloned into pcDNA3 expression vector that contains neomycin-resistant gene as selection marker for generation of the stable cell line constitutively expressing KEDPK. The cell lines we chose for stable transfection are estrogen-dependent breast cancer cell line (MCF-7) and estrogen-independent breast cancer cell line (LTSD). LTSD cell lines are kindly provided by Dr. BS Katzenellenbogen's lab which was established by long-term culture of MCF-7 cell line in the absence of steroids in the effort to study the progression of breast cancer cells from estrogen dependent to independent status. Ten micrograms of pcDNA3-KEDPK plasimd was transfected into MCF-7 and LTSD cell lines. In a parallel experiments, empty plasmid pcDNA3 was transfected into these cell lines as a negative control. Neomycin resistant clones from both lines were selected in the presence of 300 μg/ml neomycin.

Clones resistant to neomycin were isolated from MCF-7 (named MR#) and LTSD (named LR#) cell lines and expanded in the presence of neomycin. Since no antibody against KEDPK is available, the antisense probe of KRAB repression domain was generated to measure the expression of KEDPK at transcriptional level in these clones by RNase protection assay. Representative result of expression of KEDPK in clone LR2-5 is shown in **Figure 8**. The parental cell line LTSD and clone LR2-5 were treated with estrogen, RU486 or estrogen antagonist ICI 164384 as indicated. Twenty-four hours after treatment, RNA samples were isolated and hybridized with the KRAB antisense probe. The antisense cyclophilin probe was included in each sample as a loading control. As shown in **Figure 8**, KEDPK mRNA was detected only in stably transfected LR2-5 clone, not in parental LTSD cell line. The expression of KEDPK was not affected by the different hormonal treatment. Taken together, these data suggested that we established the estrogen-dependent and -independent cells stably expressing KEDPK repressor.

Functional screening of clones stably expressing KEDPK repressor by transient transfection

The effectiveness of KEDPK on ER target genes in these clones were tested by transient transfection of $(ERE)_3$tata Luc reporter. Since no endogenous ER activity was detectable under our assay condition in LTSD cell line, ER expression vector was cotransfected with the reporter gene in functional assay. Representative screening results are shown in **Figure 9**. Addition of estrogen into MR clones stimulated ER dependent transcription. ER activity was clearly inhibited by the repressor KEDPK in the presence of RU486 in these clones **(Figure 9A).** Similar results were observed in the clones isolated from estrogen-independent cell lines LTSD. Estrogen activated ER activity about 10-fold. Addition of exogenous ligand RU486 of KEDPK clearly suppressed the ER mediated luciferase activity **(Figure 9B).** Addition of RU486 had no effect on negative control cell lines transfected with empty expression vector pcDNA3 (data not shown), suggested that the inhibition of ER transcription was mediated by the stably transfected repressor KEDPK. The effectiveness of RU486-dependent inhibition of ER activity varied in different clones. The maximal RU486-dependent inhibition of ER activity in the stable cell lines is lower than that seen in transient transfection. These results indicate that the KEDPK expressed in stable cell lines functions as a RU486-dependent repressor of ER target genes.

Effect of KEDPK on the growth of estrogen-dependent and independent breast cancer cells

Since we established the estrogen dependent and independent breast cancer cell lines stably expressing KEDPK repressor and the KEDPK could effectively suppress the ER target gene expression in the presence of RU486 in transient transfection., we next tested whether KEDPK could inhibit the breast cancer cell growth dependent or independent of estrogen under these condition. MR3 and LR2-5 cells were cultured and expanded in the presence of 300 μg/ml neomycin. 1000 Cells were plated in 96 well tissue culture plates. Parental cell lines were included as negative controls. Twenty-four hours later cells were given different treatments as indicated in **Figure 10**. Growth rate of these clones were measured by MTT method on 1, 3 or 5 days after treatments. As

expected, estrogen dependent cells grow relatively slowly in the absence of estrogen. Treatment with estrogen moderately increases the growth of both MR3 and its MCF-7 cells, with more stimulating effect on MR3 cells. Under our assay condition, RU486 could slightly increase the cell growth of both MCF-7 and MR3 cells. RU486 has no significant effect on the cell growth of MR3 stimulated by estrogen. In the case of estrogen-independent cell lines, estrogen treatment slightly decreases the LR2-5 cell growth and has no effect on LTSDcell growth. Addition of RU468 slightly increases the growth of LTSD cells. Marginal decreases of LR2-5 cell growth was observed after treatment with RU486, both in the absence or presence of estrogen. The inhibitory effect of KEDPK was quite limited when compared to estrogen antagonist ICI164384 whereby almost no cell growth was observed (data not shown). These results suggested that the repressor KEDPK expressed in stable cell lines is insufficient to significantly inhibit the growth of breast cancer cells either in estrogen-dependent or independent manner.

Effect of KEDPK on endogenous ER responsive genes in breast cancer cell lines

The ability of KEDPK to suppress endogenous ER responsive genes was evaluated by RNase Protection Assay (RPA) in these breast cancer cell lines. The pS2 gene was used as an ER responsive gene marker to monitor the activity of the repressor KEDPK on endogenous ER target gene expression. RNA samples were isolated from MR3 and LR2-5 cells together with their parental cell lines 24 hrs after various treatments as indicated in **Figure 11**. The antisense probe used in RPA corresponds to the coding region of pS2 and yielded a 180 bp protected fragment. The antisense cyclophilin probe was included in each sample as a reference. Results are shown in **Figure 11A**. In estrogen dependent breast cancer cell line, E2 treatment increased the expression of pS2 in MR3 and its parental cells MCF-7. E2 antagonist ICI effectively blocked the effect of E2 on pS2 gene expression. Only 10 % inhibition of E2 induced pS2 expression was observed in the presence of RU486 in MR3 cells. Similar results were observed in estrogen-independent breast cancer cell of LR2-5 **(Figure 11B).** Very little inhibition of ER induced expression of pS2 was observed. Taken together, very limited inhibition of the expression of endogenous ER target gene pS2 was achieved by the repressor KEDPK in the presence of RU486. These results suggest that KEDPK expressed in MR3 and LR2-5 was not sufficient to inhibit the expression of endogenous pS2 ER target gene and cell growth of breast cancer cells in culture.

Alternative strategies in attempt to suppression of endogenous ER target genes

It is imperative that the repressor KEDPK suppresses endogenous ER target genes in breast cancer cell lines. It is possible that higher level of KEDPK is required to compete for binding to the promoter of endogenous ER target genes. To get around this problem, we place KEDPK into different expression vectors. KEDPK has been subcloned into episomal mammalian expression vector pCEP4 that has been shown to drive the high expression of recombinant proteins in mammalian cells. This expression could effectively inhibited ER reporter gene expression in transient transfection similar to pcDNA-KEDPK expression (Data not shown). Stable MCF-7 cell lines have been established under hygromycin selection at the concentration of 50 μg/ml. However, no significant changes of endogenous ER target gene expression were observed in these cell lines after treatment with RU486 (Data not shown).

We also attempted to combine KEDPK repression domain with histone-deacetylase (HD1). HD1 is a potent repressor of transcription, presumably by deacetylating histone in the promoter region and thus locking nucleosomes into a tight conformation that precludes access of transcription factors or maintains a suboptimum DNA structure. It is demonstrated in our lab that when fused to the transcriptionally active GAL1-147 DNA-binding domain, HD1 could repress transcriptional activity of a promoter containing GAL4 binding sites. Thus, the repressor KEDPK was modified by replacing C-terminal KRAB domain with HD1. The construct was tested in transient transfection and shows marginal inhibition of ER reporter genes in the presence of RU486 (Data not shown). Therefore, no stable cell lines were generated for this vector.

KEY RESEARCH ACCOMPLISHMENTS

1.Generated regulable repressor KEDPK of ER target genes.
2.Constructed plasmids transcribing antisense probes of specific ER responsive genes pS2 and complement component 3 (C3).
3.Established two lines of transgenic mice expressing AIB1 transgene predominantly in mammary glands.

REPORTABLE OUTCOMES

1. Ma ZQ, Tsai MJ, Tsai SY. Suppression of gene expression by tethering KRAB domain to promoter of ER target genes. J Steroid Biochem Mol Biol 1999; 69:155-63.
2. Stable MCF-7 breast cancer cell line expressing KEDPK repressor.

Conclusion

We have successfully generated regulable repressors to inhibit the ER reporter *genes in vitro* in transient transfection. Estrogen-dependent and –independent cell lines stably expressing the repressor KEDPK have been established. The repressor was functional active in these stable cell lines as demonstrated in transient transfection. However, the repressor fails to suppress endogenous ER target gene expression in breast cancer cell lines. Many factors might contribute to the inability of KEDPK to suppress the endogenous ER target genes. Among of which, the level of repressor expression might not high enough to compete with endogenous ER for regulatory elements of ER target genes. Second, even though the repressor was able to suppress the expression of ER reporter gene that is naked DNA format, the repressor was unable to get access to the regulatory region of endogenous ER target genes that is tightly compacted in nucleosome format. The ability of repressor domain to suppress reporter gene in chromatin template should be tested first in vitro so as to have better chance to generate such a chimeric transcription repressor that effectively suppresses endogenous genes in vivo.

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APPENDICES

1. Legend.
2. Figures.

Legends

Figure 1. Inducible repressor system

A. Model of inducible repressor system. The regulable repressor contains a DNA-binding domain binding to estrogen response element (ERE), a transcription repression domain obtained from Krupple-Associated Box (KRAB), and a mutated PR ligand-binding domain which responds to antiprogestin, RU486. The regulable repressor constructed in this way can compete with wild-type ER for ERE binding and turn off all the ER target genes in breast cancer cells in the presence of RU486.

B. Diagram of regulable repressor KEDPK construct.

Figure 2. In Vitro transcription/translation of the regulable repressor, KEDPK

A TNT coupled reticulocyte lysate system was used to express the KEDPK regulator. An empty expression vector and ER expression vector were included as a control. The translated products were separated in 10% SDS-PAGE gel. * denotes the expected size of translated proteins.

Figure 3. Specific inhibition of ER-dependent activation by the regulable repressor, KEDPK

Panel A. An $(ERE)_3$TATA-Luc reporter construct (100 ng) and a human ER expression plasmid (50 ng) was transfected along with or without regulable repressor, KEDPK (200 ng), into HeLa cells using lipofectin . After 6 hr of transfection, cells were washed and incubated in the presence of E_2 (1 nM) for an additional 24 hr with or without RU486 (10 nM), as indicated. The magnitude of ER activation by E_2 alone was set at 100%.

Panel B. HeLa cells were transfected with 50 ng of reporter GREtk-Luc, 50 ng of GR* (LBD truncation) with or without 100 ng regulable repressor, KEDPK. Luciferase activity was assayed 24 hrs after treatment with or without 100 nM RU486. Luciferase activity was normalized for protein quantitation.

A single experiment representative of at least two independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.

Figure 4. Inhibition of ER Activity by KEDPK in a dose-dependent manner

Cells were transfected with $(ERE)_3$TATA-Luc reporter (100 ng), human ER expression plasmid (50 ng), and an increasing amount of KEDPK repressor construct (50, 100, 200 ng). Cells were treated with 10 nM 17ß-estradiol and 100 nM RU486 for 24 hrs as indicated. The magnitude of ER activation by E_2 alone was set at 100%. A single experiment representative of three independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.

Figure 5. RU486 dose-dependent curve on KEDPK inhibition of ER activity

HeLa cells were transiently transfected with 50 ng of the $(ERE)_3$TATA-Luc reporter, 50 ng of the human ER expression plasmid, and 200 ng of the repressor construct, KEDPK. Cultures were treated with 17ß-estradiol (10 nM) and different concentrations of RU486 for 24 hrs as indicated. The magnitude of ER activation by E_2

alone was set at 100%. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.

Figure 6. The effect of KDEPK on 4-hydroxy-tamoxifen (4OH-T) stimulated ER activity

The repressor plasmid (200ng) and $(ERE)_3$TATA-Luc reporter (100 ng) were transfected together with an ER expression vector (100ng) into HepG2 (human heptocellular carcinoma) cells. Luciferase activity was normalized for quantity of protein. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.

Figure 7. The effect of KEDPK on ER mediated natural promoter activity of complement factor 3 (C3)

HeLa cells were transfected with C3-Luc promoter (100 ng), ER expression plasmid (100 ng), and KEDPK repressor construct (200ng). Cells were treated with 10 nM 17ß-estradiol and 100 nM RU486 for 24 hrs as indicated. Luciferase activity was normalized for quantity of protein. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.

Figure 8. Expression of KEDPK in transfected estrogen-independent breast cancer cell lines

RNA isolated from LR2-5 and parental LTSD cell lines 24 hours after different treatments was isolated and hybridized to KRAB and cyclophilin antisense probes. RNase analysis of KRAB transcripts was carried out according manufacturer, s instruction (Ambion). Double protected brands of KRAB shown in autoradiography are caused by minor differences of cloning size of KRAB domain between N-terminal and C-terminal.

Figure 9. Functional screening of estrogen-dependent and -independent breast cancer cell lines stably expressing KEDPK.

Breast cancer cells were transfected with pcDNA3-KEDPK using calcium phosphate precipitation. The transfected cells were selected in the presence of 300 μg/ml neomycin. At their appearance, isolated colonies of neomycin-resistant cells were taken and cultured separately.

Panel A. Clones derived from estrogen-dependent cell line MCF-7 (MR1, MR2 and MR3). 100 ng $(ERE)_3$tata-Luc reporter was transfected into the cells.

Panel B. Clones derived from estrogen-independent cell line LTSD (LR2-3 and LR2-5). 25 ng ER expression vector and 100 ng $(ERE)_3$tata-Luc reporter was transfected into the cell.

Luciferase activity was determined 24 hrs after 17β-estradiol and RU486 treatment. Results indicate that selected clones respond differently to RU486 in suppression of ER mediated transcription.

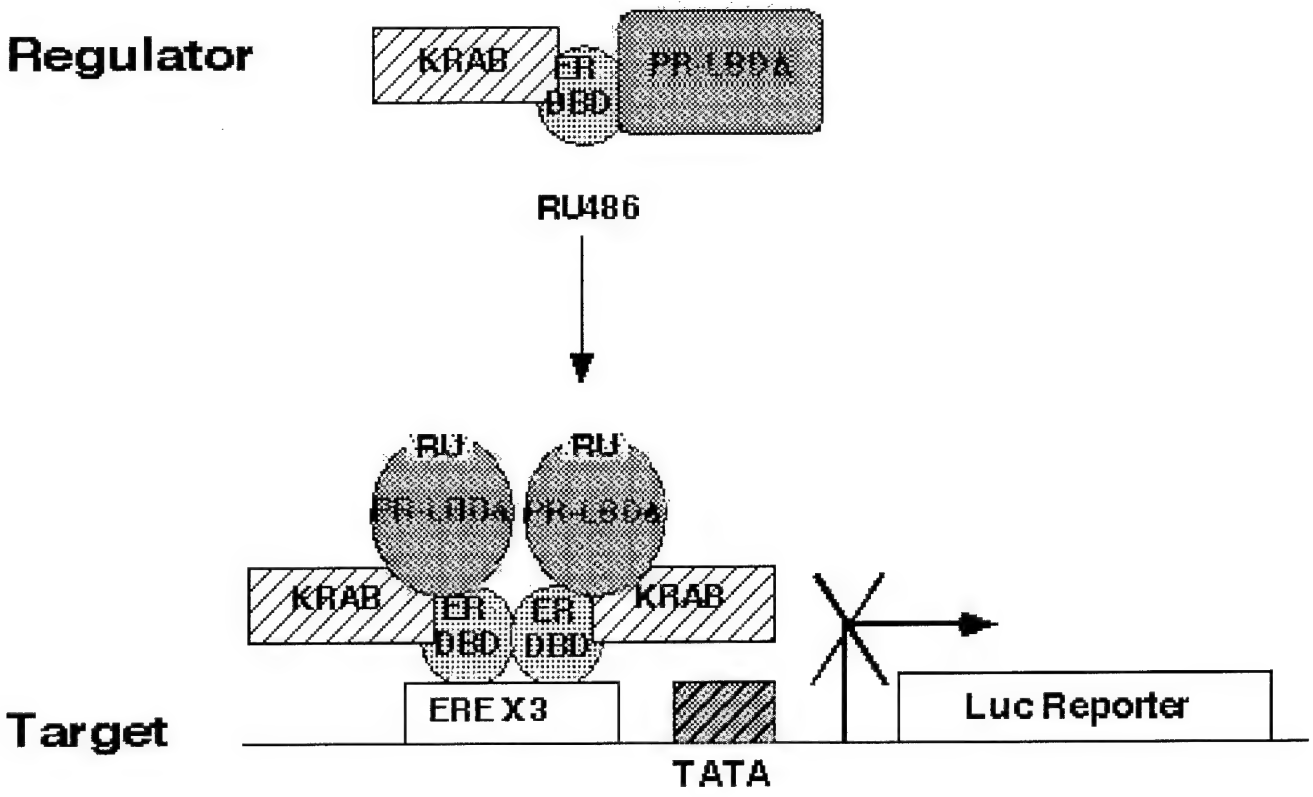
Figure 10. Effect of KEDPK on estrogen-dependent and -independent growth of breast cancer cells

Estrogen dependent MCF-7 and MR3 cells are maintained in normal DMEM+10% FCS medium, whereas estrogen independent LTSD and LR2-5 cells are cultured in DMEM+10% stripped FCS medium without phenol red. To measure the rate of cell growth, 1000 cells were plated in 96 well tissue culture plates in DMEM+10% stripped FCS medium without phenol red. 24 hour later, cells were received a different treatments as indicated in the figures. 1, 3 or 5 days after treatment, cells were treated with MTT. O.D. of each well were read in microtiter reader at 540 nm. A single experiment representative of three independent experiments is detailed above. (*: $P < 0.05$ compared to control by student *t* test, n=6)

Figure 11. Effect of KEDPK on endogenous ER responsive gene pS2 in breast cancer cell lines

RNA isolated from estrogen-dependent (MCF-7 and MR3) and estrogen-independent (LTSD and LR2-5) breast cancer cell lines LR2-5 and parental LTSD cell lines was isolated 24 hours after different treatments and then hybridized to pS2 antisense probes. Cyclophilin probe was included in each sample as loading control. RNase analysis of pS2 transcripts was carried out according manufacturer, s instruction (Ambion). Arrowheads indicate the protected fragments of pS2 and cyclophilin. Autoradiographies of RNase protection analysis were scanned and quantitated as relative optic density (relative O.D.) after normalizing to internal control. Results are drawn in left panels of their corresponding autoradiographies.

A.


Regulator
KRAB
ER DBD
PR-LBDA
RU486
RU
RU
PR-LBDA
PR-LBDA
KRAB
ER DBD
ER DBD
KRAB
Target
ERE X3
TATA
Luc Reporter

B.

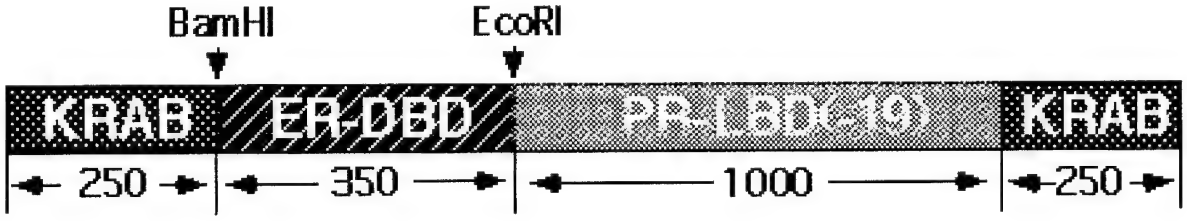

BamHI
EcoRI
KRAB
ER-DBD
PR-LBD(-19)
KRAB
250
350
1000
250

Figure 1

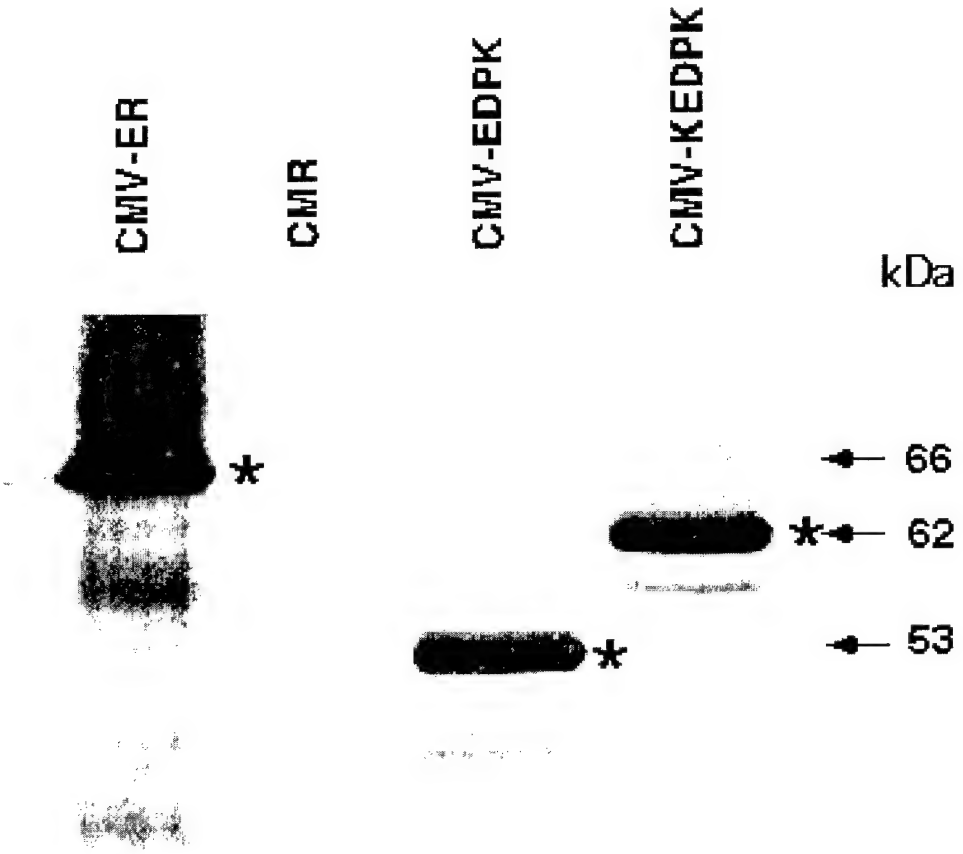


Figure 2

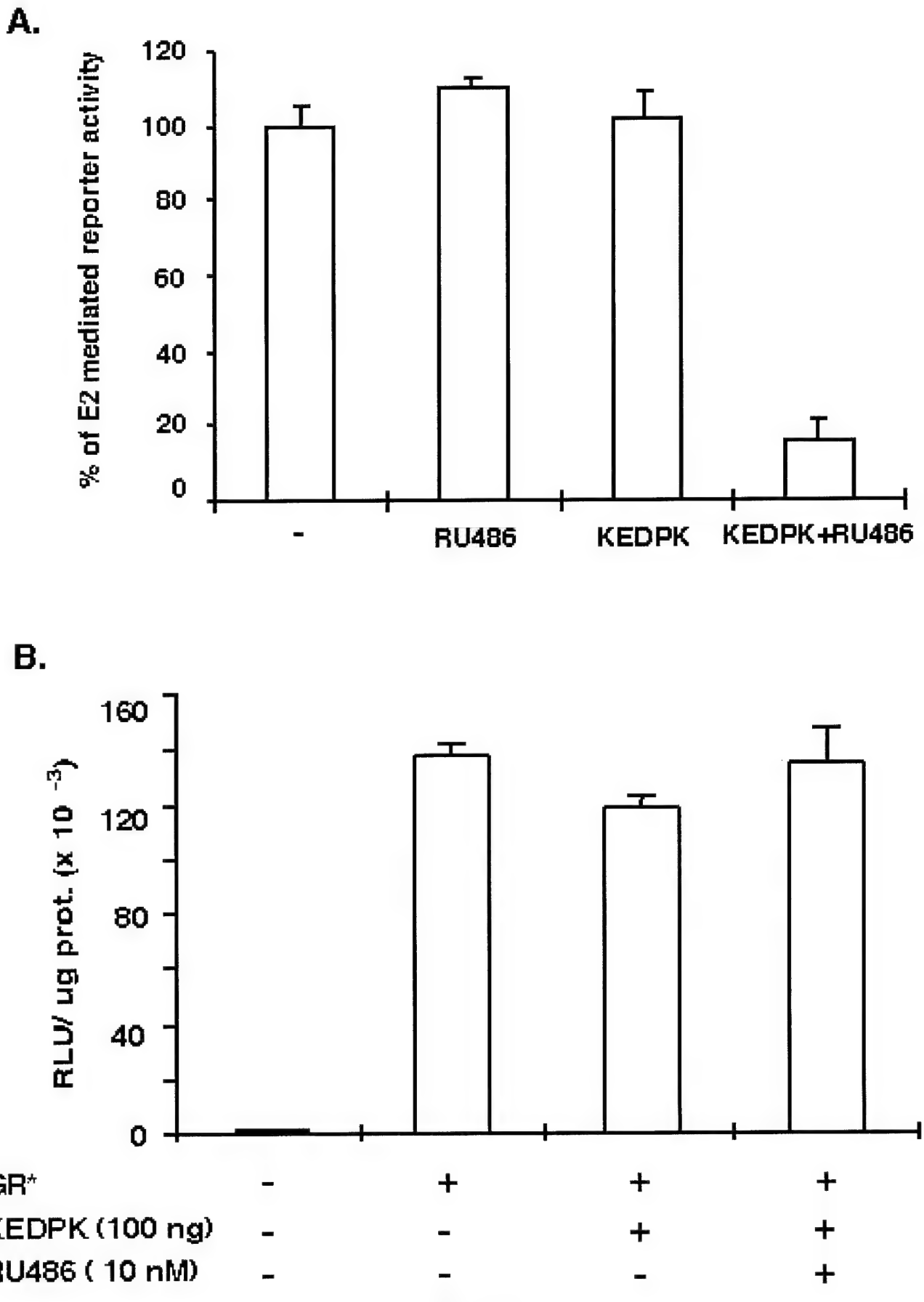

A.
% of E2 mediated reporter activity
120
100
80
60
40
20
0
-
RU486
KEDPK
KEDPK+RU486
B.
RLU/ ug prot. (x 10 -3)
160
120
80
40
0
GR*
KEDPK (100 ng)
RU486 (10 nM)

Figure 3

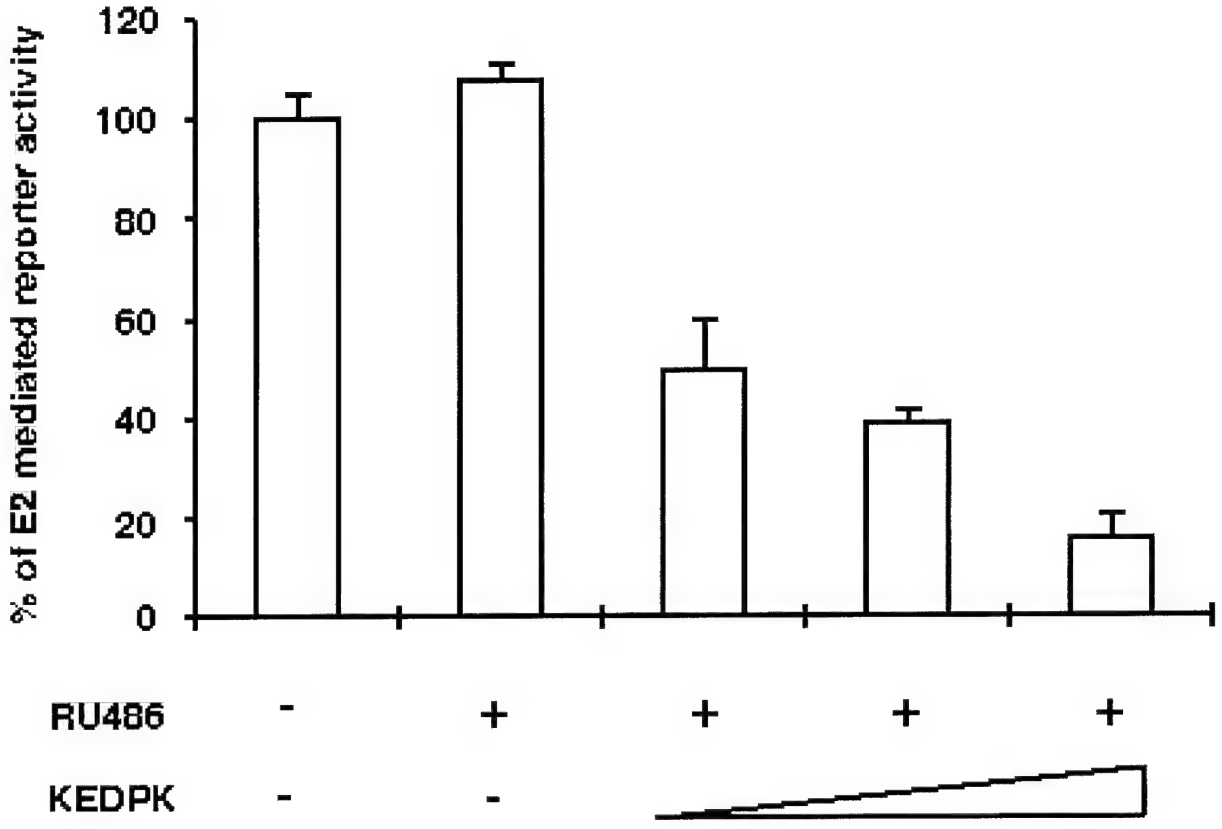

% of E2 mediated reporter activity
120
100
80
60
40
20
0
RU486
-
+
+
+
+
KEDPK
-
-

Figure 4

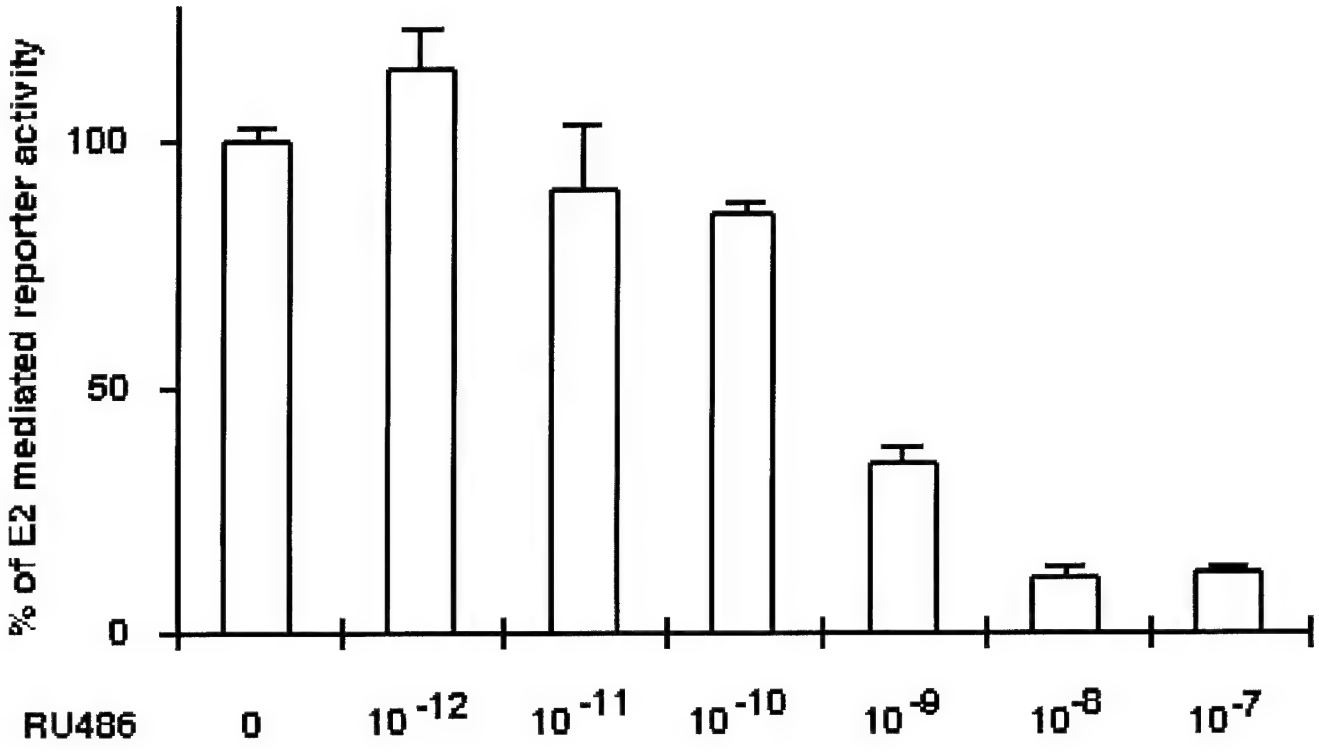

% of E2 mediated reporter activity
100
50
0
RU486
0
10 -12
10 -11
10 -10
10 -9
10 -8
10 -7

Figure 5

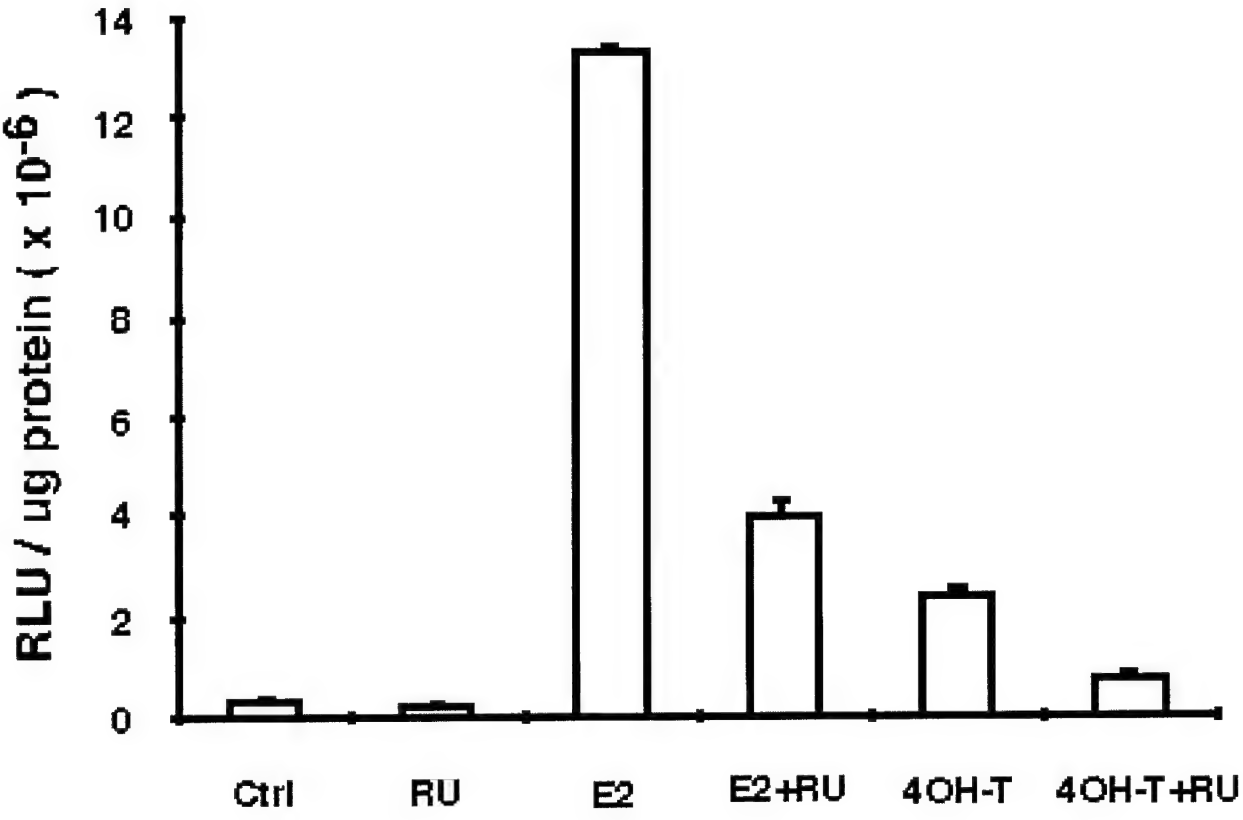


Figure 6

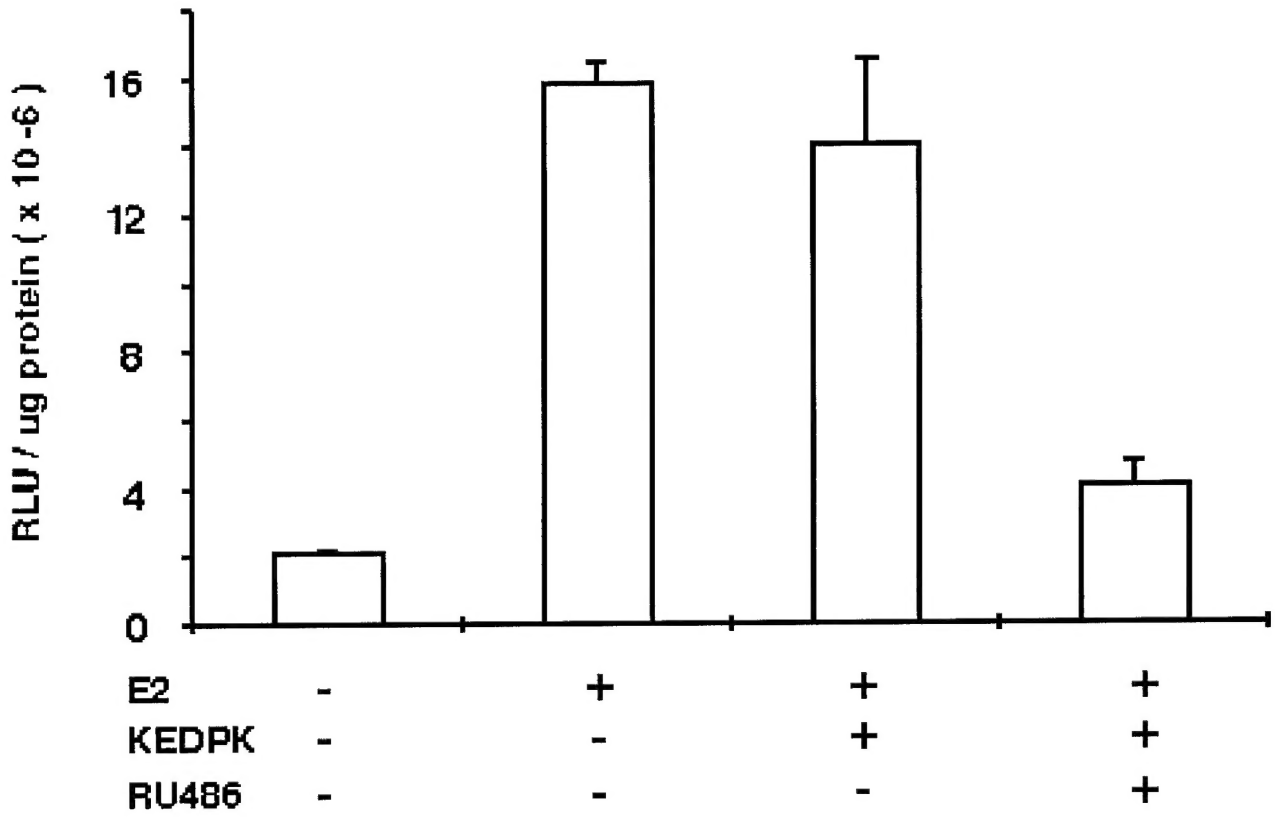


Figure 7

Functional Screening of E2-Dependnet and-Independent Breast Cancer Cell Lines Stably Expressing KEDPK

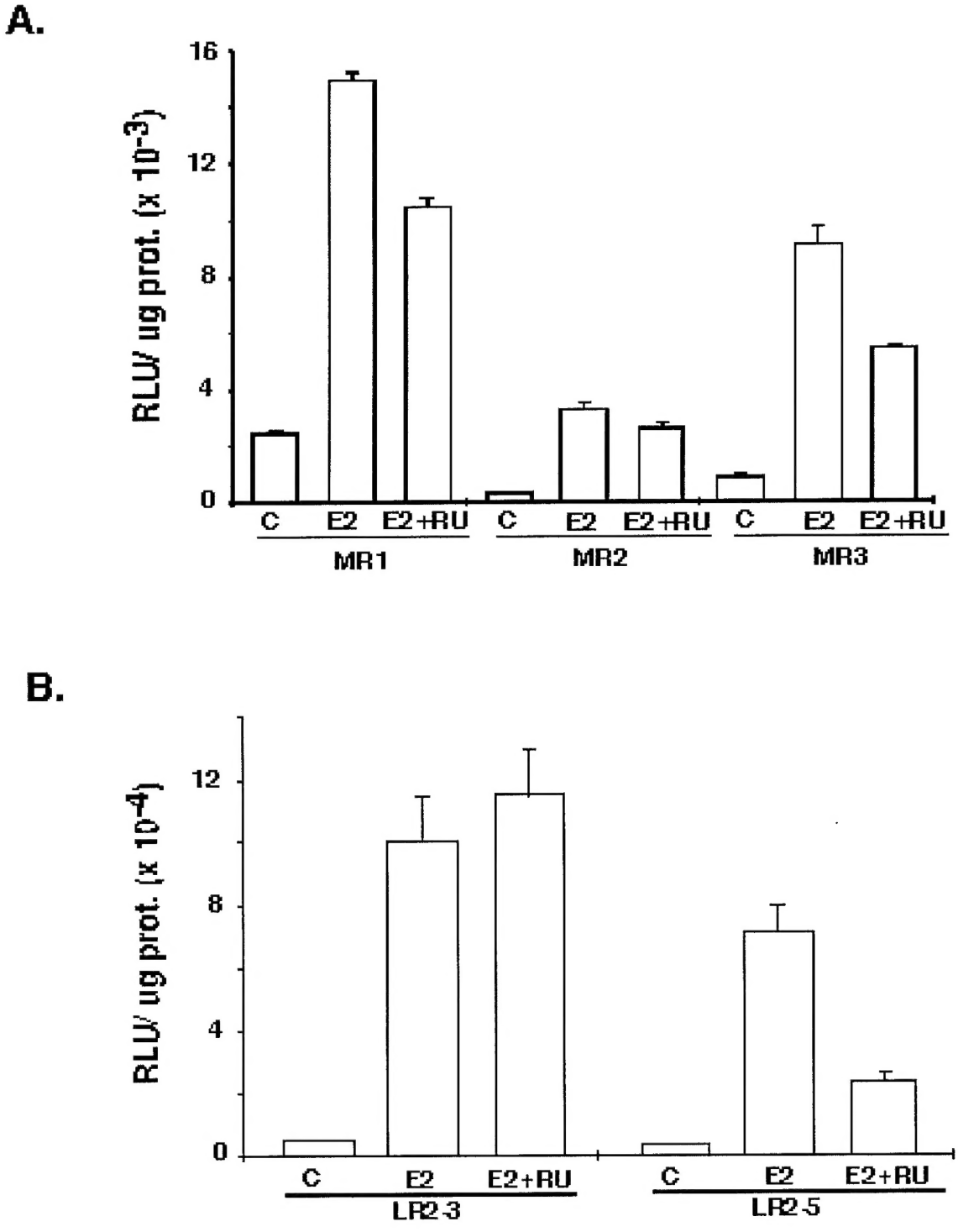


Figure 9

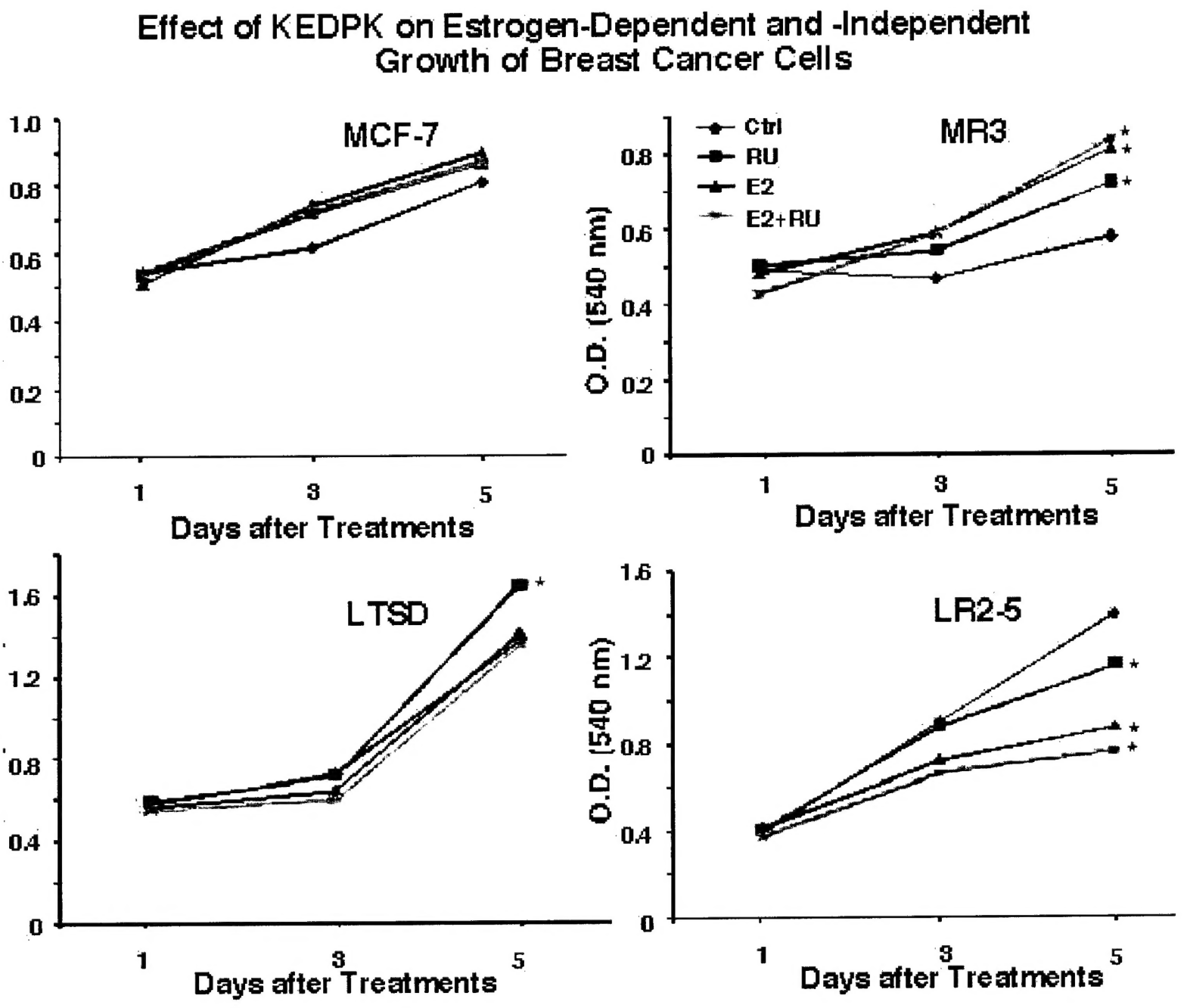

Effect of KEDPK on Estrogen-Dependent and -Independent Growth of Breast Cancer Cells
MCF-7
MR3
LTSD
LR2-5
Ctrl
RU
E2
E2+RU
O.D. (540 nm)
Days after Treatments
1.0
0.8
0.6
0.4
0.2
0
1.6
1.2
1
3
5

Figure 10

Effect of KEDPK on Endogenous ER Responsive Gene pS2 in Breast Cancer Cell Lines

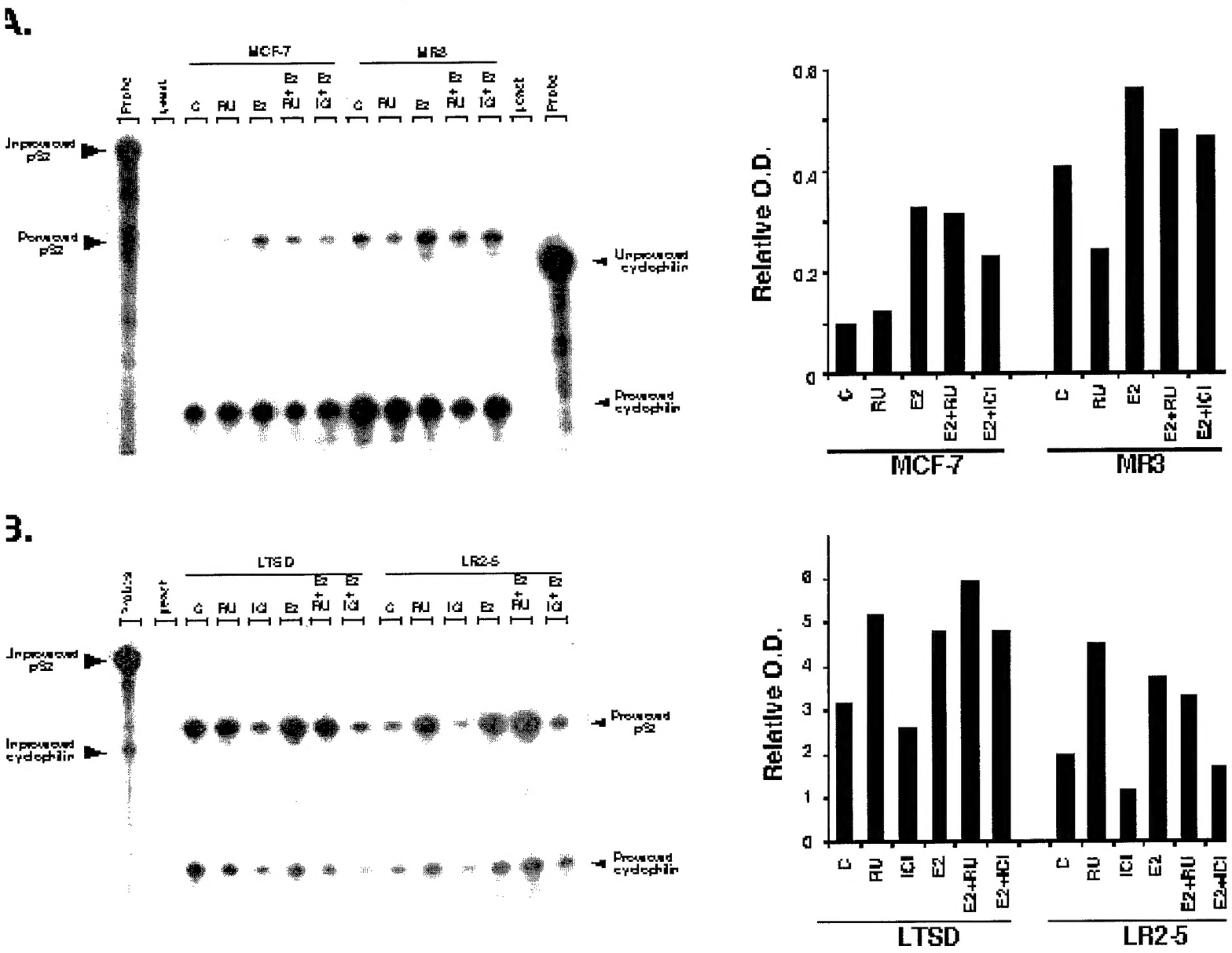


Figure 11